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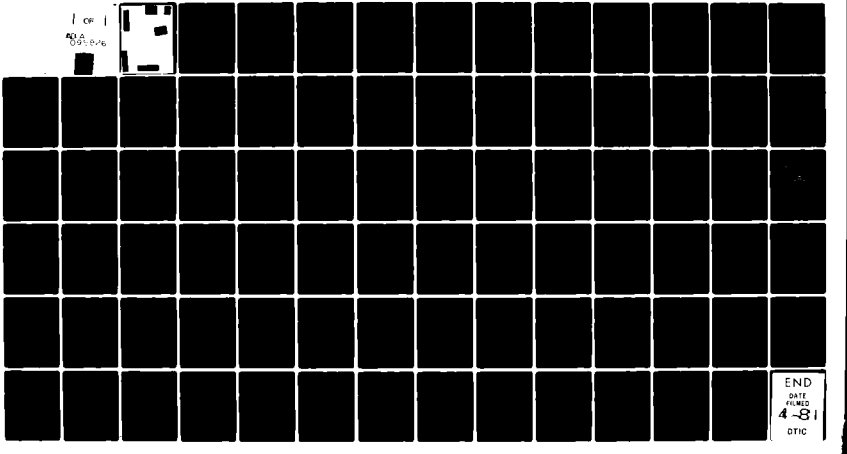

SOME OXYGEN-18 STUDIES ON THE MECHANISM OF MYO-INOSITOL OXYGENASE

1980

RICHARD L. MOSKALA

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Myo-inositol oxygenase is known to catalyze the cleavage of myo-inositol to D-glucuronic acid in the presence of O_2. Several mechanisms for this cleavage have been proposed. The present research was concerned with determining the number and location of oxygen atoms incorporated from O_2, thereby limiting the number of possible mechanisms. This was accomplished by a series of oxygen-18 labeling studies with analysis of trimethylsilylated compounds by GC/MS techniques.

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Some Oxygen-18 Studies on the Mechanism of Myo-inositol Oxygenase

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Final Report, 25 JUN 80

A thesis submitted to The Pennsylvania State University
in partial fulfillment of the requirements for the degree
of Master of Science.

The Pennsylvania State University

The Graduate School

Department of Chemistry

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A Thesis in

Chemistry

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 1980

The signatories below indicate that they have read and approved the thesis of Richard L. Moskala.

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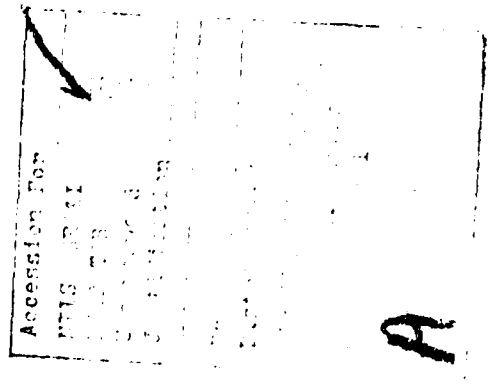
June 25, 1980	C. Robert Matthews, Assistant Professor of Chemistry, Member of Committee
June 25, 1980	Robert D. Minard, Lecturer in Chemistry, Member of Committee



ABSTRACT

Myo-inositol oxygenase is known to catalyze the cleavage of myo-inositol to D-glucuronic acid in the presence of O_2. Several mechanisms for this cleavage have been proposed. The present research was concerned with determining the number and location of oxygen atoms incorporated from O_2, thereby limiting the number of possible mechanisms. This was accomplished by a series of oxygen-18 labeling studies with analysis of trimethylsilylated compounds by GC/MS techniques.

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GLOSSARY OF ABBREVIATIONS

CI	Chemical Ionization
EDTA	Ethylenediaminetetraacetic Acid
EI	Electron Impact
GC/MS	Gas Chromatograph / Mass Spectrometer
GSH	Glutathione
HTP	Hexamethyldisilazane, Trimethylsilyl chloride, Pyridine
m/z	Mass/Charge Ratio
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
RIC	Reconstructed Ion Chromatogram
SDS	Sodium Dodecylsulfate
TCA	Trichloracetic Acid
TMS	Trimethylsilyl
TPNH	Triphosphopyridine Nucleotide (reduced form)

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I. INTRODUCTION

A. General

1. Purpose

The present research is concerned with the mechanism of the enzymatic oxidation of myo-inositol to D-glucuronic acid. Although F. C. Charalampous reported that one atom of oxygen-18 is incorporated from $^{18}O_2$ during the cleavage of myo-inositol, he could not conclusively state where the label appears and whether one or two atoms of oxygen-18 are incorporated (1). Current techniques in gas chromatography/mass spectrometry now permit the cleavage mechanism to be further elucidated. Specifically, the main aspects of this research will be to determine (a) if the incorporation of oxygen-18 occurs in the carboxyl of D-glucuronic acid or in the aldehyde site and (b) how many atoms of oxygen-18 are actually incorporated. A study of this type could limit the number of possible mechanisms.

To avoid oxygen-18 exchange at the aldehyde site, it is advantageous to reduce D-glucuronic acid to L-gulonic acid. Since a constituent enzyme, D-glucuronic acid reductase, is present in the crude stages of myo-inositol oxygenase purification (2), it was decided not to purify the oxygenase to homogeneity. Therefore, a crude extract containing both myo-inositol oxygenase and D-glucuronic acid reductase was prepared and L-gulonic acid

was produced as the end product. This research was accomplished by evaluating L-gulonic acid with appropriate labels and comparing the enzymatic oxidation-reduction system to the models. The overall reaction scheme is shown in Figure 1.

2. Background

Myo-inositol oxygenase is found in both plant and mammalian systems. In 1957, F. C. Charalampous reported that an enzyme system in rat kidney extracts was capable of cleaving inositol to glucuronic acid (3). A year later he isolated and purified the enzyme, myo-inositol oxygenase, responsible for this cleavage (4). Hoffmann-Ostenhoff, et al. have shown that myo-inositol is converted to D-glucuronic acid by an oxidase in a yeast (5). Shortly afterwards, F. A. Loewus presented evidence that myo-inositol metabolism in parsley (Petroselium) leaves and strawberry (Fragaria) fruits proceeded through a D-glucuronic acid pathway (6). More recently, Hoffmann-Ostenhoff isolated the enzyme from oat seedlings (7). In this laboratory, Dr. C. Channa Reddy has purified and characterized myo-inositol oxygenase from pig kidney (8). Investigations have continued with the further characterization of myo-inositol oxygenase, especially in the areas of metal content and cofactors necessary for the enzymatic cleavage.

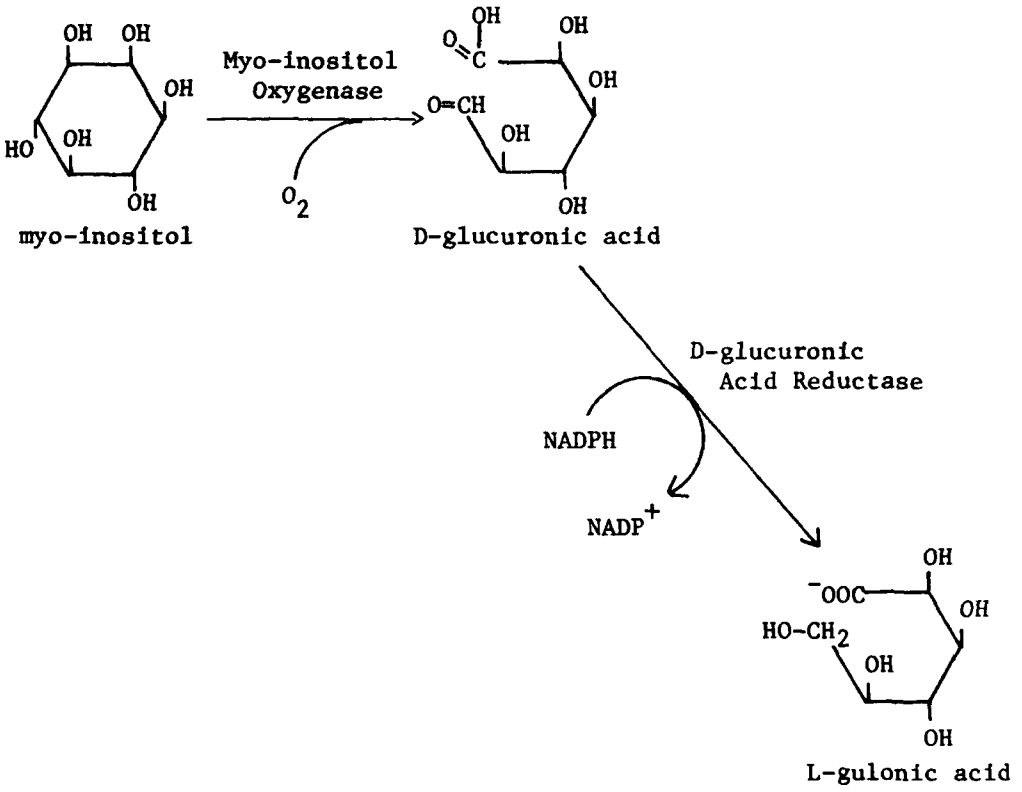
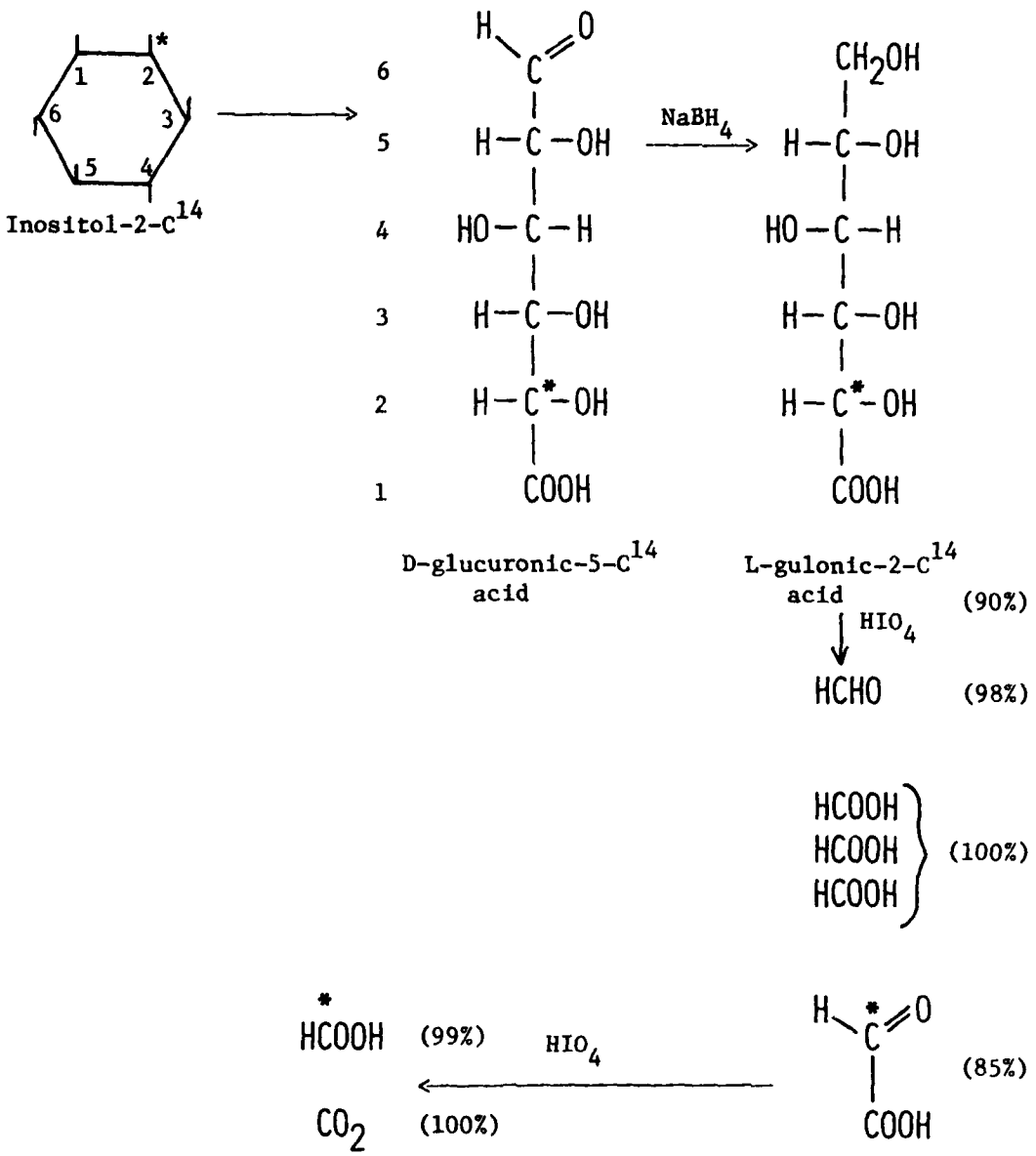


Figure 1: Overall Enzymatic Oxidation-Reduction Reactions.

B. Proposed Cleavage Mechanisms

F. C. Charalampous identified the cleavage site of myo-inositol (9) by employing inositol-2-C^{14} as substrate. D-glucuronic acid was reduced to L-gulonic acid and chemically degraded with periodic acid. More than 98.5% of the C^{14} was recovered in the glyoxylate fraction. Further oxidation of this fraction to carbon dioxide and formic acid resulted in all radioactivity located in the formate, as shown in Figure 2. Thus, Charalampous concluded that myo-inositol oxygenase cleaves myo-inositol between C-1 and C-6 to form exclusively the D-glucuronic acid. In addition, Charalampous proposed that the mechanism of cleavage of myo-inositol occurred through an enzyme-oxygen-enediol complex (1). He suggested that myo-inositol first loses two hydrogen atoms from C-1 and C-6 to form the enediol. This is followed by cleavage of the double bond and fixation of one atom of oxygen to form the carboxyl of glucuronic acid. The two hydrogens removed from inositol combine with one atom of oxygen to form water. This pathway is illustrated in Figure 3.

However, Posternak found this mechanism to be impossible. In a series of deuterium labeling studies, he determined that myo-inositol labeled at C-6 with deuterium gives rise to a deuterium at C-1 in D-glucuronic acid (10). In glucogenesis from D-glucuronic acid, the deuterium atom is carried through to the final end product, glucose. Posternak expected the isotope to appear



The numbers indicate the carbon atoms of inositol and the figures in parentheses indicate the yields at each step.

Figure 2: Chemical Degradation and Distribution of C^{14} in D-glucuronic Acid Derived from Enzymatic Cleavage of Inositol-2-C^{14} (1).

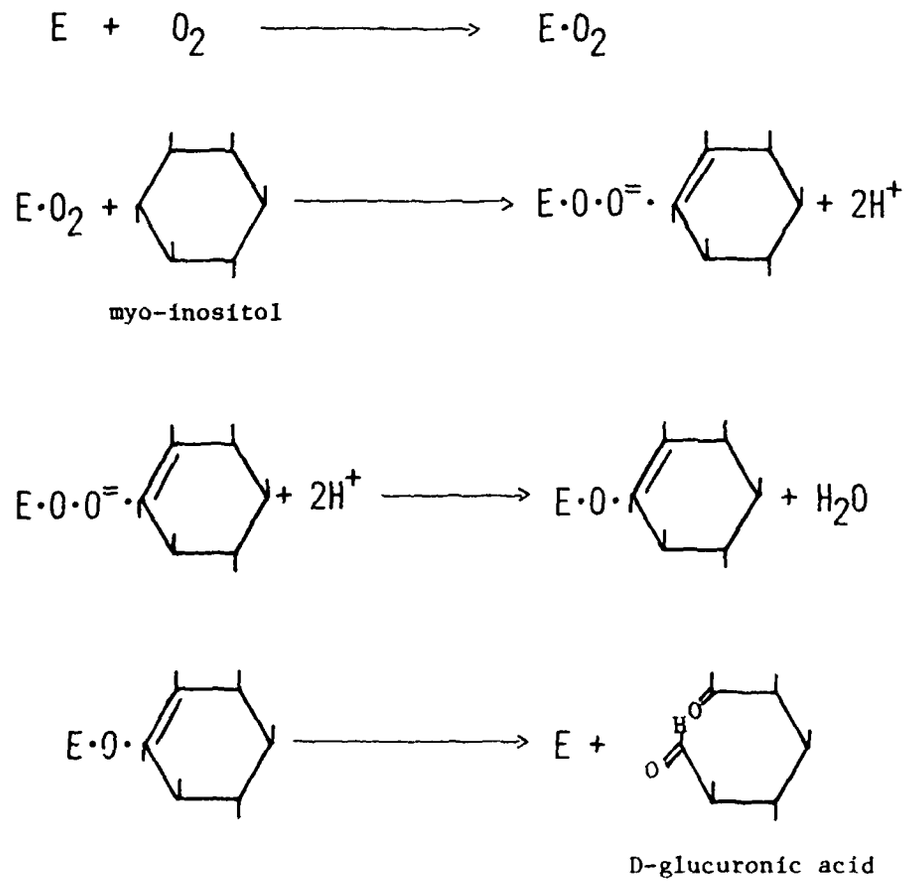
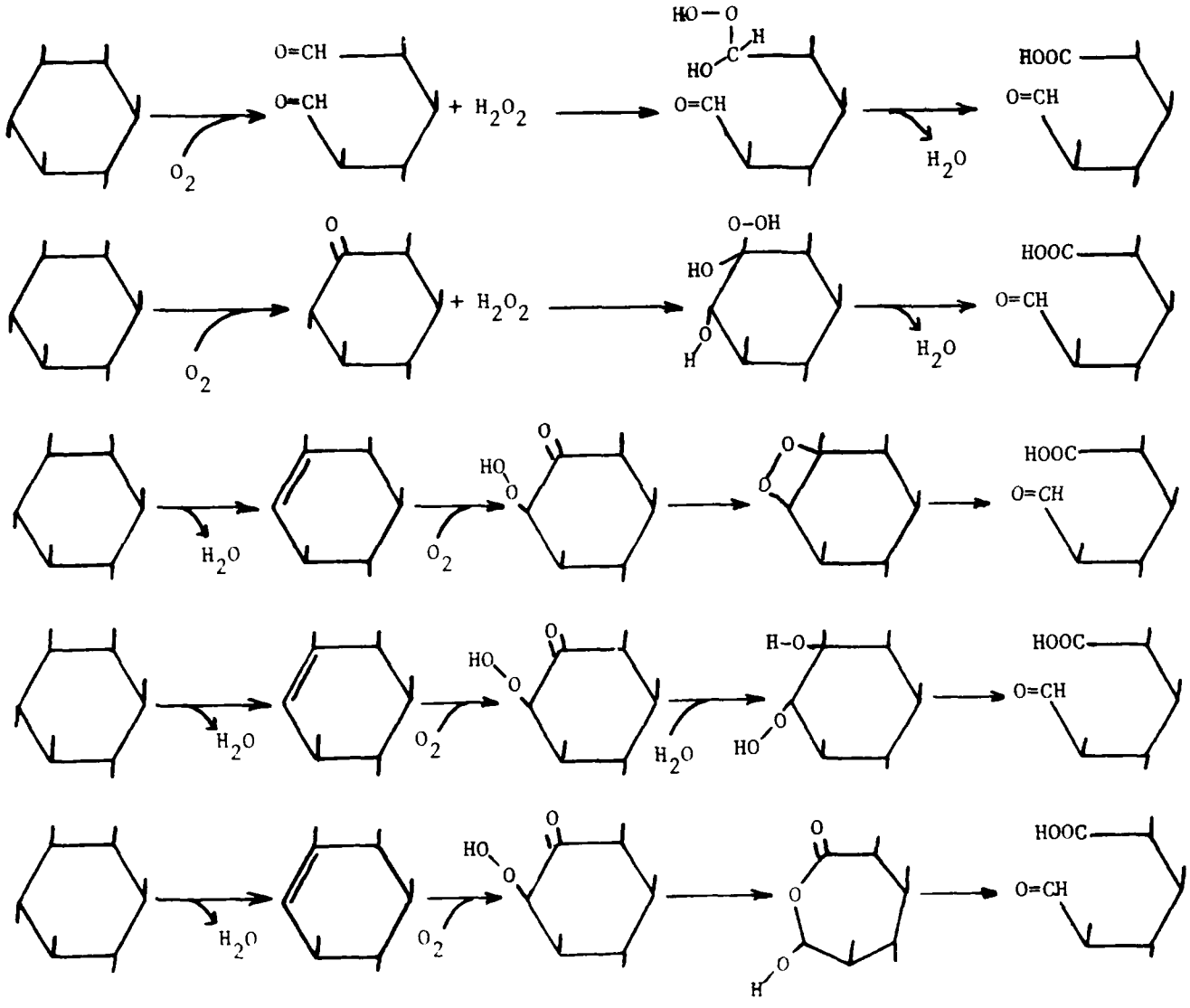


Figure 3: Charalampous Proposed Mechanism for *Myo-inositol Cleavage* (1).

primarily in the C-1 position of glucose with some deuterium appearing at C-3. Experimental results confirmed this theory (11).

Dr. Gordon A. Hamilton has proposed several mechanisms for the enzymatic cleavage of myo-inositol to D-glucuronic acid. These are represented in Figure 4. Several of these mechanisms can be eliminated by appropriate oxygen-18 studies. By converting the D-glucuronic acid to L-gulonic acid, the possibility of oxygen-18 exchange with solvent at the aldehyde position is minimized. Trimethylsilylation of L-gulonic acid and subsequent analysis by GC/MS provides an analytical method in locating the exact site of oxygen-18 incorporation and the number of oxygen-18 atoms incorporated. This is in contrast to Charalampous' oxygen-18 work (1), where the oxygen-18 labeled glucuronic acid was pyrolyzed and then analyzed for oxygen-18 enriched carbon dioxide. To further define Dr. Hamilton's proposed mechanisms, an oxygen-18 model study of L-gulonic acid must first be accomplished. Oxygen-18 can be incorporated in the carboxyl of L-gulonic acid by the saponification of L-gulono-γ-lactone in the presence of oxygen-18 enriched water. The B_{ac2} mechanism assures that the label will appear only in the carboxyl group. Identification of the mass spectral fragments containing oxygen-18 would be indicative of the carboxyl group. Similarly, if oxygen-18 were to exchange at the aldehyde site of D-glucuronic acid, the compound could be chemically reduced to L-gulonic acid and analyzed by GC/MS for oxygen-18 containing fragments. L-gulonic acid, produced by the enzymatic oxidation-reduction

Figure 4: Hamilton's Mechanisms for Myo-inositol Cleavage to D-glucuronic Acid.


MECHANISM
I
II
III
IV
V
O=CH
HOOC
HO—O
HO
O-OH
H-O
O_2
H_2O_2
H_2O

reaction in the presence of $^{18}O_2$, could then be analyzed by the same techniques for oxygen-18 enriched fragments and compared to the model studies. If the comparison shows that no oxygen-18 is incorporated in the aldehyde site, Dr. Hamilton's mechanisms III, IV, and V could be eliminated. Similarly, if oxygen-18 enrichment is found in the carboxyl group (mechanisms I and II), the number of oxygen-18 atoms present would be valuable in further defining the mechanisms.

C. Characteristics of Myo-inositol Oxygenase

1. Average Molecular Weight

The average molecular weight of myo-inositol oxygenase from rat kidney has been reported by Charalampous (4) to be 68,000. This calculation was based on sedimentation-diffusion analysis (74,000), the approach to sedimentation equilibrium method (66,000), and the iron content of the enzyme (63,000). Channa Reddy (8) determined the molecular weight of the enzyme from pig kidney to be 65,000 from both SDS gel electrophoresis and sedimentation equilibrium. The gel filtration method produced a similar molecular weight. The molecular weight of the myo-inositol oxygenase from oat seedlings (7) was found to be 63,000 by gel electrophoresis.

2. Specific Activity

The specific activity reported in the literature for myo-inositol oxygenase varies according to the source. Charalampous (4) found the purified enzyme to have a specific activity of 106

mKatals/Kg. However, Channa Reddy measured a lower activity of 21.9 mKatals/Kg for the homogeneous enzyme isolated from pig kidney which required cysteine and Fe^{++} for activity (8). Hoffmann-Ostenhoff reported 950 μKatals/Kg for the oat seedling product (7).

3. Michaelis Constant and Optimum pH

The effect of substrate concentration on enzymatic activity was studied by Charalampous. By the Lineweaver-Burk method he calculated a Km value of 2.21×10^{-2} M at an optimum pH of 6.8 - 7.1 (4). Hoffmann-Ostenhoff (7) determined the apparent Michaelis constant to be 4.3×10^{-3} M at pH 7.2. Channa Reddy (8), however, calculated the Km value for inositol oxygenase in a cysteine/Fe^{++} system to be 5×10^{-3} M. This was accompanied by a sharp optimum pH at 6.0.

4. Iron Content

In studying inhibition of the enzyme by various chelating agents, Charalampous' results suggested the presence of a metal as a part of inositol oxygenase. Accordingly, bound nonhemin iron was found to be present. The ratio of the specific activity to the iron content is constant during the last stages of purification. Charalampous calculated that the iron content is approximately 1 atom of iron per molecule of protein (4). Mr. J. Swan of this laboratory, however, calculated the iron content to be 4 atoms per molecule assuming a molecular weight of 65,000. Less active

preparations of the enzyme apparently contained less than 4 atoms of iron (12). Although Hoffmann-Ostenhoff did not analyze the enzyme for iron content, he concluded that iron was an essential component from other data (7).

5. Inhibition of Inositol Oxygenase

Charalampous (4) found that metal binding agents such as cyanide (5 mM), azide (1.3 mM), and 8-hydroxyquinoline (3.5 mM) inhibit enzymatic activity. Additionally, he reported that sulfhydryl reagents such as p-chloromercuribenzoate, phenylmercuric nitrate, and arsenite cause up to 90% inhibition at mM or less concentration. Of all the organic inhibitors tested, only p-chloromercuribenzoate, phenylmercuric nitrate, and cyanide could be blocked by glutathione. Charalampous also reported that metals, particularly Cu^{++}, Ag^{+}, and Hg^{++}, which form mercaptides with the enzyme's -SH groups are potent inhibitors at 10^{-4} M. As with the organic inhibitors mentioned above, this effect could be prevented by the addition of glutathione or cysteine. Hoffmann-Ostenhoff (7) reported inhibition of the oat seedling derived enzyme by sodium cyanide, 8-hydroxyquinoline, and p-chloromercuribenzoate at similar concentrations. However, unlike the rat kidney inositol oxygenase, ATP and NAD ($NADP^{+}$) have no effect on the enzyme activity. Complete inhibition of the cysteine/Fe^{++} system by Fe^{+++}, Cu^{++}, and Hg^{++} with mM amounts was reported by Channa Reddy (8). Interestingly. he also reported that chelators known

for specificity towards copper also cause inhibition on the order of 80% or greater.

D. Characteristics of D-glucuronic Acid Reductase

1. Background

In 1957, A. L. Lehninger reported that a TPNH-specific dehydrogenase which reduces D-glucuronic acid is present in rat kidney extract; in fact, to a greater extent than liver extracts (13). Since then, the literature has made references to TPN-L-gulonate dehydrogenase, TPN-L-hexonate dehydrogenase, TPNH-linked aldehyde reductase, and D-glucuronate reductase--all derived from pig kidney extracts.

2. Molecular Weight

Bosron and Prairie (14) reported an average molecular weight of 30,000 by conventional sedimentation equilibrium for the TPN-linked aldehyde reductase. SDS gel electrophoresis gave a slightly higher estimate (38,000) and sephadex G-200 gel filtration produced a much lower molecular weight (25,000). However, Walton and coworkers (15) estimated a molecular weight of 33,000 by Sephadex G-100 gel filtration and 36,900 by SDS gel electrophoresis.

3. Michaelis Constant

The enzyme catalyzes reversibly both oxidation and reduction of the aldehyde and alcohol groups of the substrate and product. Bosron and Prairie (14) calculated an apparent Km for D-glucuronate to be

8.4×10^{-3} M. Similarly, Bublitz, et al. (16) listed a Michaelis constant of 7.2×10^{-3} M for L-gulonate.

4. Inhibition of D-glucuronate Reductase

Bublitz (16) found that the enzyme was completely inhibited by 10^{-3} M p-chloromercuribenzoate. However, this was reversed with the addition of GSH implying the need for intact sulfhydryl groups. On the other hand, Bosron and Prairie (14) reported less than 10% inhibition at 10^{-3} M concentrations of p-hydroxymercuribenzoate, 2-mercaptoethanol, iodoacetamide, and EDTA. Of possible physiological importance, however, they found 85% inhibition at 1 mM by the barbiturate, amobarbital.

II. EXPERIMENTAL SECTION

Unless otherwise noted, only doubly distilled and deionized water was used in the preparation of all buffers and in the conduct of any experimental procedure in this research. Distilled water was first passed through an Ultrapure Mixed Bed ion exchange column (Barnstead catalog number D0809) and then through an Organic Exchange column (Barnstead catalog number D8904). The deionized water was then distilled a second time in a Kontes WS-2 Continuous Water Still (glass distillation apparatus).

A. Materials

1. Buffers

Sodium phosphate buffer was prepared as outlined by Gomori (17). All pH measurements were taken on a Fisher Accumet pH meter (model #144) with a Fisher Microprobe Electrode (catalog # 13-639-92), or on a Radiometer type TTTlc equipped with a scale expander type PHA 630T and a type GK2402C electrode.

Monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$), dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), and potassium chloride were obtained from Mallinckrodt. GSH was purchased from Sigma. All were at least reagent grade or better and used without further purification.

2. Substrates

Myo-inositol and D-glucuronic acid were obtained from Sigma and used without further purification.

3. Reagents and Miscellaneous Compounds

All commercially available materials were reagent grade or better and used without further purification. Sodium hydroxide, concentrated hydrochloric acid, and trichloroacetic acid were obtained from J. T. Baker. Ferric chloride, cysteine·HCl, and sodium acetate were purchased from Aldrich. NADPH (reduced form, Type III) and orcinol were procured from Sigma. Sodium fluoride and chloroform were bought from the Fisher Scientific Company. Ferrous ammonium sulfate was purchased from Mallinckrodt and ultra-pure ammonium sulfate was obtained from Schwartz/Mann.

Sylon HTP was purchased from Supelco, Inc. of Bellefonte, PA. This reagent, packaged in 0.1 ml ampules, contained hexamethyl-disilazane, trimethylchlorosilane, and pyridine in a 3:1:9 ratio. L-gulono-γ-lactone (mp 183-4°) was obtained from ICI. Sodium borohydride was purchased from Alfa Products (Ventron). A mixed ion exchange resin, MB-3, was obtained from Rohm and Haas Co., Philadelphia, PA. Absolute ethanol was provided by Publicker Industries, Inc., also of Philadelphia.

Oxygen-18 enriched water was obtained from Bio-Rad. The water used contained 20.1% and 20.0% enrichment. Molecular oxygen-18 gas with 90% isotopic purity was purchased from Merck Isotopes.

B. Analytical Methods

1. Gas-Liquid Chromatography

Prior to submitting any sample for analysis, the solid was thoroughly dried in a dessicator over drierite at a reduced pressure. The sample was then trimethylsilylated according to the method outlined by Sweeley and Wells (18). Approximately 1-2 mg of each solid was treated with 0.1 ml of HTP and shaken vigorously for 30 seconds. Although no attempt was made to remove the white precipitate, centrifugation was helpful if the precipitate was copious.

Gas-liquid chromatograms were obtained on a Finnigan 9500 Gas Chromatograph with a flame ionization detector. Helium was used as the carrier gas with a flow rate of 20 cc/min. Separation of the components was achieved isothermally at 160°C with a 5 ft. glass column packed with 3% OV-17. Detector and injector temperatures were set at 250°C.

2. Mass Spectrometry

All mass spectra and related data were obtained on a Finnigan 3200 Gas Chromatograph/Mass Spectrometer equipped with a Finnigan 6000 Data System. Electron impact at 70 electron volts with a quadrapole mass analyzer was used most frequently. However, the chemical ionization method was needed to produce the molecular ion (parent) peak. In these cases, methane was used as both the reagent gas and the carrier gas. Gas chromatograph conditions were the same as those discussed above.

Two techniques were used to determine the amount of oxygen-18 incorporation or exchange. In both cases, the intensities of various ions were measured as well as the intensities of those ions 2 amu higher. The intensities for an ion of interest were integrated across the entire gas chromatograph peak in lieu of using the intensity from a single spectrum. The first technique involved recording total spectra (m/z 100-650) continuously every 1-2 seconds and storing them in the computer system. After the trimethylsilylated compounds had eluted, the computer was programmed to produce traces of a specific ion obtained from each complete scan throughout the entire chromatographic run. These traces are called reconstructed ion chromatograms (RIC). The area of each RIC could then be calculated by the computer. Ratios of these peak areas (m/z + 2 : m/z) could then be compared. The second technique, called single ion monitoring, consisted of monitoring continuously the intensities of only four ions instead of scanning the entire mass range. Signal to noise ratio and sensitivity are greatly improved because essentially all of the time is spent integrating only the signals from the four specific ions and no others. Areas of these single ion chromatograms could then be measured and evaluated in a manner similar to the first technique.

3. Colorimetric Assay of Myo-inositol Oxygenase Activity

The standard assay mixture (2.0 ml) modified by Channa Reddy (8) contained 50 mM sodium phosphate buffer at pH 7.2, 2.0 mM cysteine, 1.0 mM ferrous ammonium sulfate, enzyme solution

(0.2 ml) and water to make the volume 1.5 ml. The contents were incubated for 5 minutes at room temperature before the addition of 0.5 ml of 240 mM myo-inositol. The assay mixture was then incubated at 30° ±0.5°C in a Dubnoff Metabolic Shaking Incubator for 15 min. The reaction was quenched with the addition of 0.1 ml of TCA (1 g/1 ml), shaken on a vortex mixer, and centrifuged. The supernatant was added to 5.0 ml of orcinol reagent (180 mg $FeCl_3$ and 800 mg orcinol per 200 ml conc. HCl). The solutions were shaken vigorously and placed in a warm water bath (90°C) for 30 min. After cooling for 30 min, the optical density of each assay was measured at 660 nm with a Gilford 240 Spectrophotometer with a 1 cm quartz cell. The instrument was blanked with doubly distilled water.

All specific activities were reported in Katals/Kg in accordance with IUPAC/IUB recommendations (19). A Katal is defined as that amount of enzyme which catalyzes the formation of 1 mole per second.

4. Assay of D-Glucuronic Acid Reductase Activity

The reductase was assayed spectrophotometrically by monitoring the consumption (oxidation) of NADPH at 340 nm in a Gilford 240 Spectrophotometer with a 1 cm quartz cell. The standard assay mixture (1.0 ml) developed by Channa Reddy (2) contained 50 mM sodium phosphate buffer at pH 7.2, 0.2 mM NADPH, enzyme solution (0.1 ml) and water to make the volume 0.9 ml.

At this point the recorder was adjusted to maximum absorbance. The assay was substrate initiated by the addition of 0.1 ml of D-glucuronic acid and rapid mixing of the contents in the cell was done to insure linear rates. Specific activity is reported in Katals/Kg.

5. Determination of Protein Concentration

The crude protein concentration was determined spectrophotometrically using the UV absorption method of Warburg and Christian (20). Optical density at 260 nm and 280 nm was measured with a Gilford 240 Spectrophotometer.

C. Preparation of Crude Enzymes

In the isolation of pure myo-inositol oxygenase and pure D-glucuronic acid reductase, all of the initial steps developed by Channa Reddy (2,8) are the same. Therefore, the purification process of the two enzymes was halted in the crude stage before separation of the enzymes occurred.

1. Preparation of Crude Extracts

Pig kidneys were obtained from the Juniata Meat Packing Company, Tyrone, PA. Within ten minutes after the animals were killed, the kidneys were immediately chilled in ice. All further operations were performed at 4°C. The kidneys were minced and washed (3 x) with ice cold water within one hour of the slaughter. The minces were homogenized in a Waring blender for 30 sec with

3 volumes (weight/volume) of 25 mM sodium phosphate buffer, pH 7.0, containing 1 mM GSH and 50 mM KCl. Excess foam was carefully wiped off. The extract was then swirled with a Pasteur pipette to remove excess fat and simultaneously fluxed with nitrogen gas. The kidney extract was further homogenized in a Potter-Elvehjem Teflon Homogenizer. The resultant homogenate was centrifuged at 20,000 x (11,000 rpm) for 30 min in a Sorvall RC 2B centrifuge using a GSA-3 rotor. The pellet was discarded and ferrous ammonium sulfate was added to the supernatant to a final concentration of 1 mM. The solution was then fluxed with nitrogen gas. The extract was centrifuged again at 140,000 (30,000 rpm) for 45 min in a Beckman Ultracentrifuge L-50 using a rotor type 35. The reddish-pink supernatant was referred to as UCS.

2. Ammonium Sulfate Fractionation

Solid ammonium sulfate was slowly added to the enzyme solution (UCS) to give a final concentration of 30%. After all the ammonium sulfate had dissolved, the pH of the solution was adjusted to 7.0 with dilute ammonium hydroxide. The suspension was stirred for 1 hour, followed by centrifugation at 20,000 x g for 10 min. The precipitated protein was discarded and the resulting supernatant was brought up to 45% saturation with the slow addition of solid ammonium sulfate. The suspension was stirred again for 1 hour. The contents were then centrifuged at 20,000 x g for 10 min. The supernatant was carefully drained off. The pellets were washed

with 65% ammonium sulfate solution and then gently resuspended in 25 mM sodium phosphate buffer, pH 7.2, containing 1 mM GSH and 50 mM KCl. This suspension was dialyzed for 9 hours against 32 volumes of its buffer. The dialyzed solution was centrifuged for 30 min at 20,000 x g to remove any insoluble material. The supernatant, designated ASD, was assayed for enzyme activity and then frozen in 10 ml aliquots.

D. Oxygen-18 Labeling Procedures

1. Hydrolysis of L-Gulono-γ-lactone

A standard solution was prepared by dissolving 0.018 g of L-gulono-γ-lactone and 0.005 g of sodium hydroxide in 0.5 ml of water. The resulting concentrations were 250 mM base and 200 mM lactone. Identical amounts of the gulonolactone and sodium hydroxide were dissolved in 0.5 ml of water enriched with 20.1% oxygen-18. Both solutions were placed in a Dubnoff Metabolic Shaking Incubator at 37° $\pm$ 0.5°C. After 24 hours, the pH of the solutions was adjusted to 7.35 with dilute acetic acid. The sodium salt of L-gulonic acid was then isolated in a procedure similar to the Rehorst and Naumann method (21). Absolute ethanol (2.0 ml) was added to 0.2 ml aliquots of each solution. The solutions became cloudy immediately upon the addition of the alcohol, and were stored at -10°C for 3 days. After removal from the freezer, the samples were centrifuged and the supernatants discarded. The solid sodium salts were immediately placed in a vacuum dessicator

over drierite and thoroughly dried at reduced pressure. After silylation, the standard gulonic acid and the oxygen-18 labeled acid were analyzed by GC/MS techniques previously discussed.

2. ^{18}O Exchange with $H_2{}^{18}O$ and Subsequent Reduction of D-Glucuronic Acid

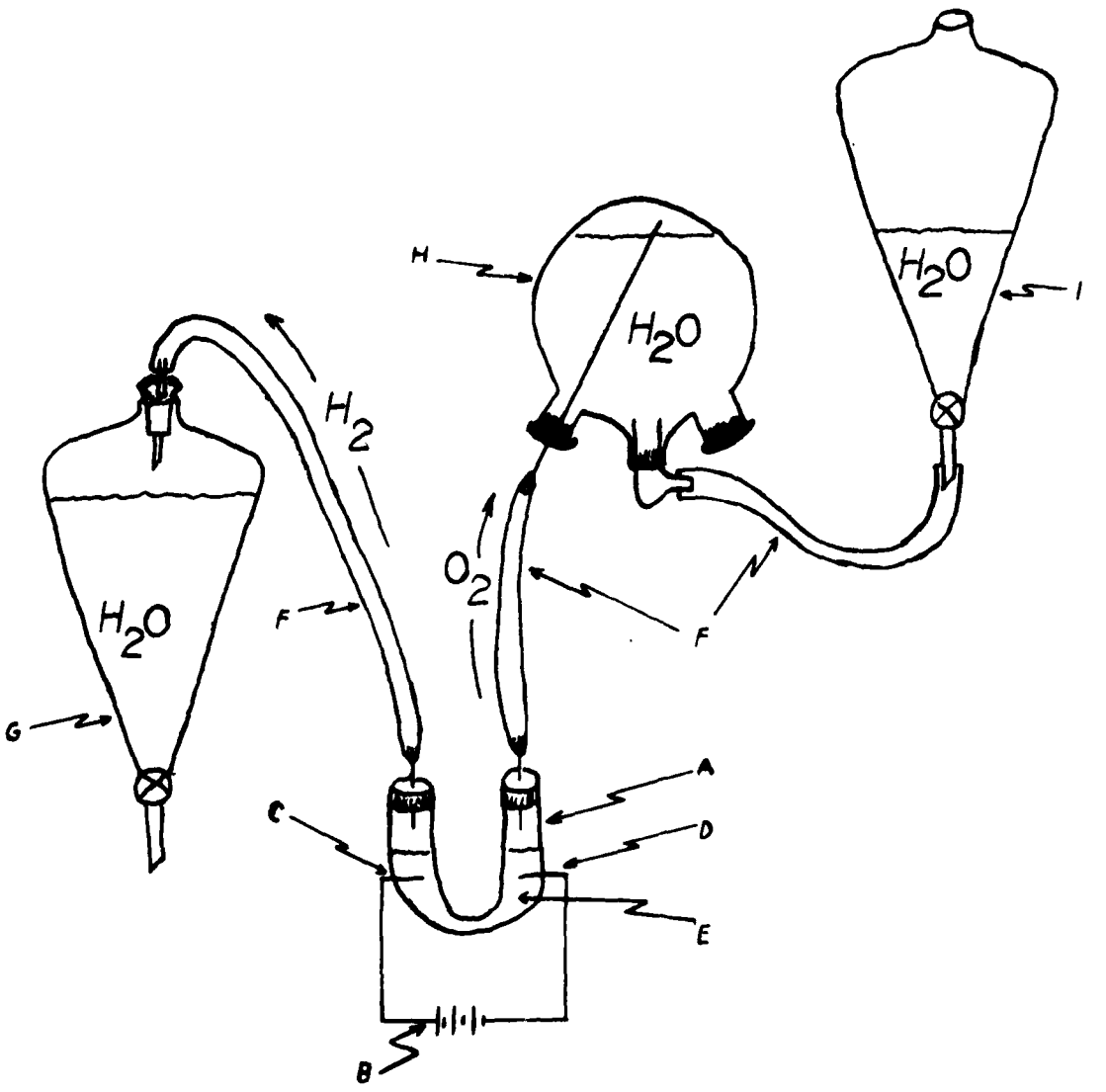
In a procedure similar to that of Sweeley and Wells (18) and performed at room temperature, D-glucuronic acid (9.8 mg) was dissolved in 0.5 ml of 50 mM sodium phosphate buffer, pH 7.1, prepared with 20.1% oxygen-18 enriched water. In preparing the buffer, exact amounts of the sodium phosphate reagents were weighed out and dissolved in 0.5 ml of oxygen enriched water. It is recognized that some ^{16}O comes from the sodium phosphate reagents, but this amounts to less than 1.0% contamination. Aliquots (0.1 ml) of the acid/buffer solution were reduced with 1.0 mg of $NaBH_4$ at internals of 0, 1, 5, 20, and 60 min. Excess $NaBH_4$ was removed with the addition of 0.1 ml of 1N HCl. When bubbling had stopped, 0.2 ml of H_2O was added. The solution was passed through 200 mg of a mixed ion exchange resin, MB-3. The resin was washed once with 0.5 ml of H_2O and the washing was added to the solution. Each sample was evaporated to dryness in a warm water bath under a stream of air. Absolute ethanol (5.0 ml) was added to each residue and the samples were stored at -10°C for 24 hours. After centrifugation, the supernatants were discarded and the solid gulonate was dried in a vacuum dessicator at reduced pressure.

A control solution was prepared in an identical manner with two exceptions. Normal 2x distilled water was used for the buffer preparation and a single 0.1 ml aliquot was reduced with $NaBH_4$ after 1 min. All samples were analyzed by GC/MS techniques.

In an attempt to force oxygen-18 exchange at the aldehyde position, 3.4 mg of sodium acetate was dissolved in both 0.5 ml of 20.1% oxygen-18 enriched water and 0.5 ml of 2x distilled water. The buffers were made acidic (pH 4.8) with concentrated glacial acetic acid. D-glucuronic acid (9.8 mg each) was added to the buffers. The two samples were placed in a warm water bath (60°C) and stirred for 24 hours. Aliquots (0.2 ml) of each reaction were reduced and isolated with the above procedure.

3. Generation of Oxygen-18 Enriched Molecular Oxygen

Approximately 30-40 ml of oxygen-18 labeled oxygen gas was generated in an 8 hour period. This was accomplished by the electrolysis of 1.0 ml of water enriched with 20.0% oxygen-18. Sodium fluoride (1 M) was used as a supporting electrolyte. The apparatus used for this procedure is presented in Figure 5 and was designed by M. Fraser (22). A Heath Kit (0.15 v, 0-500 mamp) was used as the DC power source. Prior to the addition of the enriched water to the electrolysis cell, the entire system was flushed with nitrogen gas. The $^{18}O_2$ generated was collected over water and used as soon as possible.

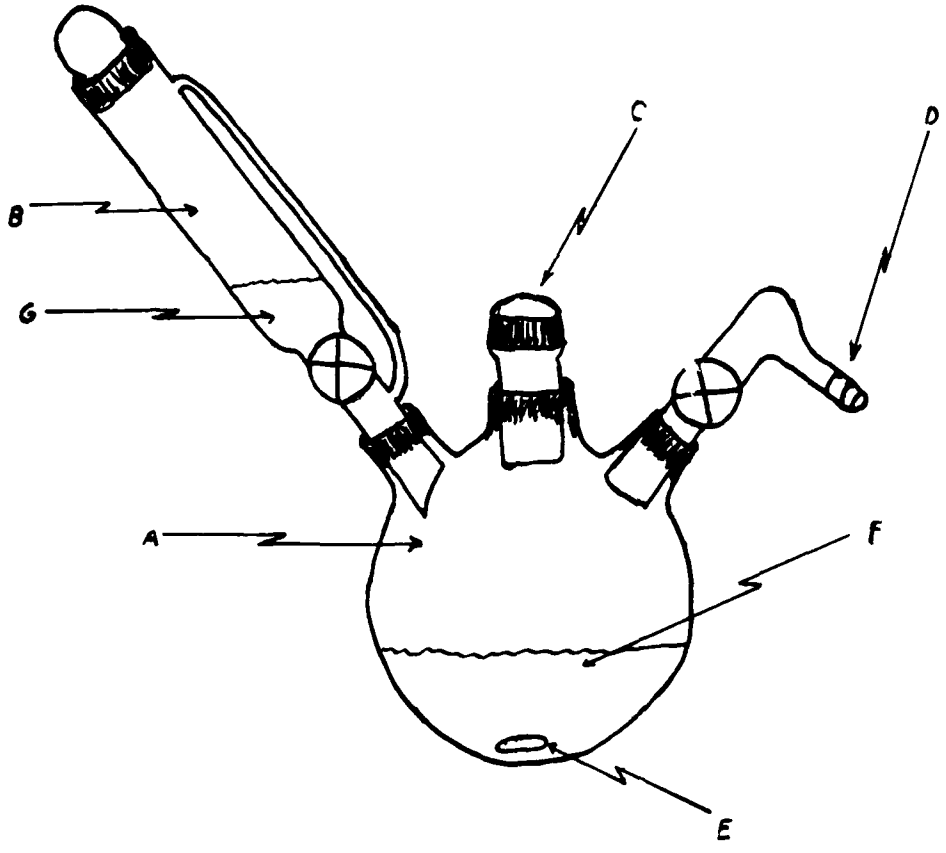


A. Electrolysis cell made from drawn out 8 mm glass tubing, 3 ml vol
B. DC power source
C. Cathode, Pt electrode, 1.5 cm
D. Anode, Pt electrode, 1.5 cm
E. H_2O with 1 M NaF, 1.0 ml vol
F. Connecting tubing, tygon
G. H_2 pressure release
H. Collection flask, 100 ml 3-necked round bottomed flask
I. Leveling flask

Figure 5: Electrolysis Apparatus (22).

4. Enzymatic Oxidation-Reduction of Myo-inositol

A 10.0 ml crude enzyme solution was thawed and placed in a 100 ml, 3-necked round bottomed flask. Myo-inositol (1.2 ml of 100 mM solution--final concentration 10 mM) was placed in the addition funnel (see Figure 6). The reaction vessel was then evacuated and sealed. In a preliminary run, approximately 30-40 ml of $^{18}O_2$ gas (generated by above procedure or commercially prepared) was injected into the flask by a gas-tight syringe. However, in the final runs with 90% $^{18}O_2$, the 100 ml $^{18}O_2$ gas container was placed between the reaction vessel and the vacuum pump by means of a 3-way stopcock. After evacuation of the reaction vessel, the vacuum pump was closed off and the $^{18}O_2$ allowed to enter. A 40 mM cysteine/20 mM Fe^{++} solution (0.6 ml) was added with slow stirring of the contents. The substrate was then released into the enzyme solution. Within 30 sec, NADPH (0.2 ml of 120 mM solution) was injected into the flask. After 20 min of stirring at room temperature, the contents were boiled for 5 min in a hot water bath and then removed from the flask. Prior to opening the reaction vessel, the $^{18}O_2$ was collected in its 100 ml container by cooling the container with liquid N_2, thereby allowing a second enzymatic reaction in $^{18}O_2$ to be accomplished immediately after completion of the first run. The protein was precipitated with an equal volume of chloroform. Following centrifugation, the aqueous layer was decanted and the precipitation by chloroform repeated. The aqueous layer was removed and adjusted to pH 7.2 with dilute NaOH. This solution was then placed in a 50 ml



A. 100 ml, 3-necked round bottomed flask
B. Addition funnel
C. rubber septum
D. Vacuum pump and $^{18}O_2$ container connection
E. Stir bar
F. Enzyme solution
G. Substrate

Figure 6: Reaction Vessel for the Oxidation-Reduction of Myo-inositol.

Amicon Ultrafiltration Cell (Model #52) and 50 psi of nitrogen gas applied. A 3.0 ml aliquot of the filtrate was reduced in volume to approximately 0.2 ml by a rotovap condensor. Absolute ethanol (6.0 ml) was added to the sample. The solution was stored at $-10^{o}C$ for 24 hours. The sample was then centrifuged and the supernatant discarded. The solid was thoroughly dried in a vacuum dessicator at reduced pressure before GC/MS analysis.

A standard, using normal isotopic oxygen, was also prepared according to the above procedure. However, the reaction vessel was not evacuated and the reaction was conducted open to the air.

III. RESULTS

A. General

In the analysis of the polysilylated compounds by mass spectrometry, only a few ion fragments were of significance. A. E. Pierce (23) presented several commonly occurring fragments of silyl ethers. These are summarized in Table 1 and are identified by the mass/charge ratio (m/z). Although these common silicon-containing ions appear in the mass spectra of polysilylated gulonic acid with various intensities, they had to be ignored as these ions are not necessarily unique to the oxygen-18 labeled site(s) of L-gulonic acid. Thus, the remaining TMS fragments were more useful in the analysis even though most occurred with lower abundance (relative intensity). The EI generated mass spectrum of 1,2,3,-4,5,6-hexakis-O-(trimethylsilyl)-L-gulonic acid is shown in Figure 7. This spectrum shows fragments up to m/z 450 while later spectra produced even larger fragment ions. Structures for the various ion fragments are proposed in Table 2. The common fragments listed in Table 1 are omitted.

Pierce also noted that the GC absolute retention time of a TMS derivative for a given stationary phase and temperature is poorly reproducible (24). Therefore, the labeled gulonate was always compared with its unlabeled standard. Tables 3 and 4 present absolute retention times of the poly-TMS gulonic acid and poly-TMS

Table 1: Common Fragments of Polysilyl Ethers

m/z	Structure
73	Me_3Si^+
75	$\overset{+}{H}O{=}SiMe_2$
89	$CH_2{=}\overset{+}{O}SiHMe_2$
103	$CH_2{=}\overset{+}{O}SiMe_3$
129	$Me_3Si\overset{+}{O}{=}CH{-}CH{=}CH_2$
147	$Me_2Si{=}\overset{+}{O}SiMe_3$
177	$Me_2Si{=}\overset{+}{O}{-}CH_2{-}OSiMe_3$
191	$Me_3SiOCH{=}\overset{+}{O}SiMe_3$
204	$(Me_3SiOCH{=}CHOSiMe_3)^+$
217	$Me_3SiOCH{=}CH{-}CH{=}\overset{+}{O}SiMe_3$
305	$Me_3SiOCH{=}C(-OSiMe_3)CH{=}\overset{+}{O}SiMe_3$

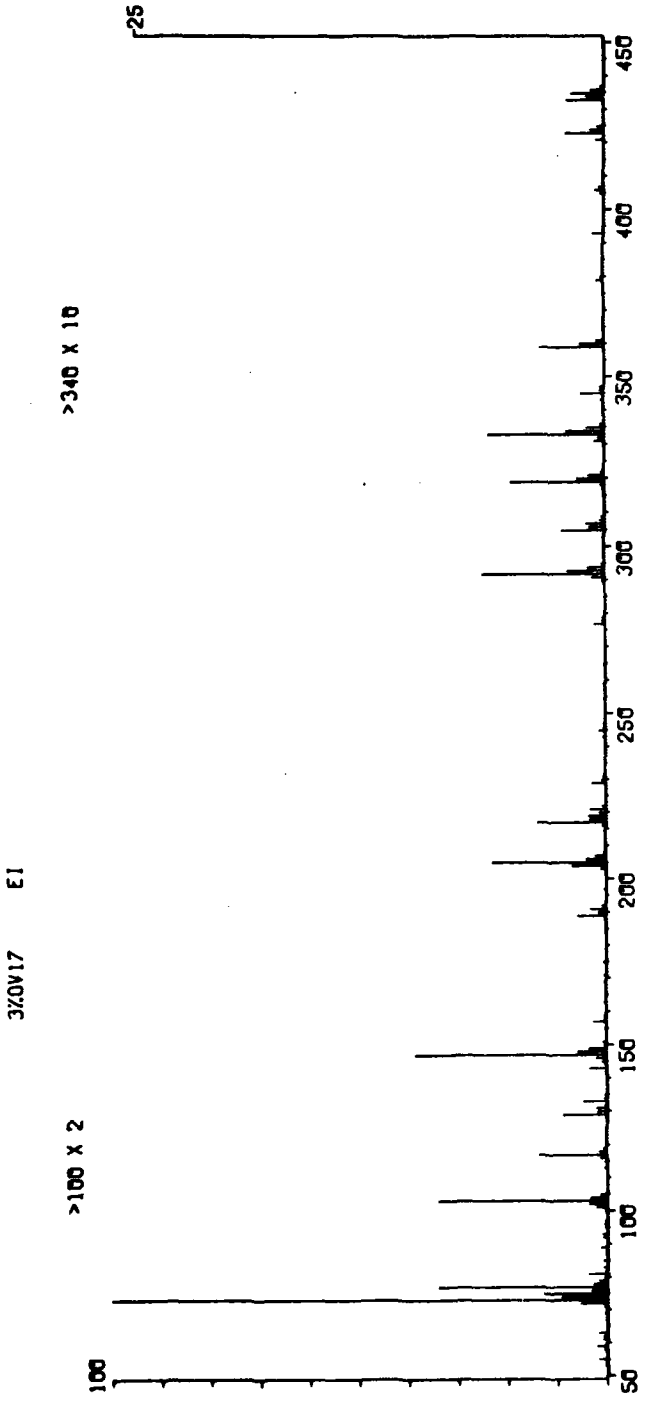


Figure 7: EI Spectra of 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-L-gulonic Acid.

Table 2: Proposed Fragments of 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-L-gulonic acid

m/z	Proposed Structure
117	$Me_2Si{=}\overset{+}{O}{-}CH_2CHO$
133	$Me_3SiO{-}CH_2{-}CH{=}\overset{+}{O}H$
205	$Me_3SiO{-}CH_2{-}CH{=}\overset{+}{O}{-}SiMe_3$
292	$[Me_3SiO{-}CH{=}C(-OSiMe_3)_2]^+$
307	$Me_2Si{=}\overset{+}{O}{-}CH_2{-}CH(-OSiMe_3){-}COOSiMe_3$
319	$Me_3SiOCH_2{-}C(=\overset{+}{O}{-}SiMe_3)CH{=}CHOSiMe_3$
333	$Me_3SiO{-}CH{=}CH{-}C(=\overset{+}{O}SiMe_3){-}COOSiMe_3$
345	$CH_2{=}CH{-}C(-\overset{+}{O}{=}SiMe_2){=}CH{-}CH(-OSiMe_3){-}COOSiMe_3$
359	$Me_3Si\overset{+}{O}{=}CH{-}CH{=}C(-OSiMe_3){-}CH{=}CH{-}COOSiMe_3$
423	$Me_3Si\overset{+}{O}{=}CH{-}CH(-OSiMe_3){-}CH(-OSiMe_3){-}COOSiMe_3$
435	$Me_3SiO{-}CH{=}CH{-}C(=\overset{+}{O}{-}SiMe_3){-}CH(-OSiMe_3){-}COOSiMe_3$

Table 3: Absolute Retention Times of TMS L-gulonic Acid and Myo-inositol

Date	Experiment	Sample ID	Peak ID	Retention Time (minutes)
8-10-79	Saponification	Test Run (a)	Gulonate	4.63
			Gulonate	5.30
12-20-79	Saponification	Test Run	Gulonate	7.13
			Gulonate	8.88
2-7-80	^{18}O Exchange at aldehyde Psn	Control-1 (b)	Gulonate	10.25
			Gulonate	10.88
		^{18}O Lab x 5 min (b)	Gulonate	10.31
			Gulonate	10.94
3-12-80	ID of poly-TMS compounds	Inositol	Inositol	5.50
			Inositol	8.25
			Inositol	10.63
		Salt	Gulonate	4.88
			Gulonate	6.38
		Inositol/Salt	Gulonate	6.13
			Inositol	6.44
			Gulonate	7.81
			Inositol	9.38
			Inositol	12.00

Conditions: Isothermal @ 160°C with 3% OV-17 Column prepared 12-10-79.

Notes: (a) 3% OV-17 date prepared unknown.
(b) Temperature plot; initial 100°C, final 250°C; rate 8°C/min.

Table 4: Absolute Retention Times of TMS L-gulonic Acid and Myo-inositol: Enzymatic System

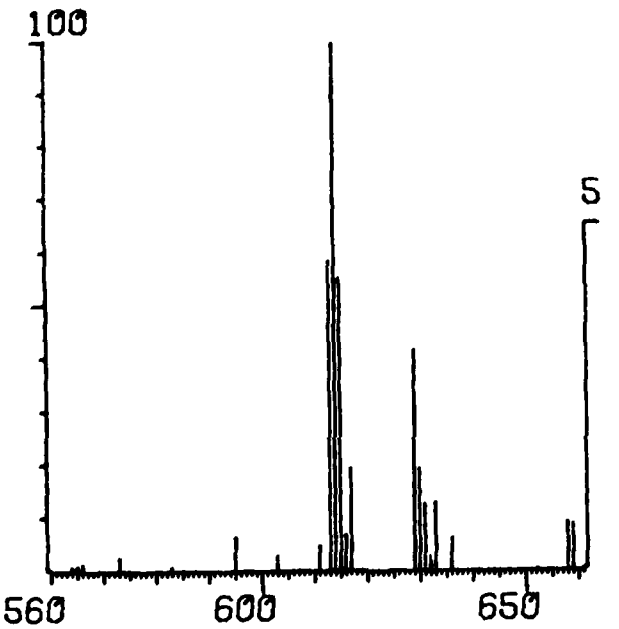
Date	Sample ID	Peak ID	Retention Time (minutes)
5-14-80	Enzyme-2 (a)	hexa-TMS-gulonate	6.50
		penta-TMS-inositol	7.25
		hexa-TMS-inositol	11.50
			13.38
			14.00
5-23-80	Enzyme-Standard (b)	hexa-TMS-gulonate	7.75
		penta-TMS-inositol	8.44
		hexa-TMS-inositol	12.63
			15.13
			16.00
	Enzyme-^{18}O Label (b)	hexa-TMS-gulonate	7.56
		penta-TMS-inositol	8.38
		hexa-TMS-inositol	12.63
			15.06
6-6-80	Enzyme-Standard (c)	hexa-TMS-gulonate	5.81
		penta-TMS-inositol	6.30
		hexa-TMS-inositol	9.75
			12.25
	Enzyme (90%) ^{18}O Label (c)	hexa-TMS-gulonate	5.69
		penta-TMS-inositol	6.25
		hexa-TMS-inositol	9.44
			11.31

(a) Isothermal @ 160°C with 3% OV-17 column prepared 12-10-79.
(b) Isothermal @ 160°C with 3% OV-17 column prepared 5-8-80.
(c) Isothermal @ 160°C with 3% OV-17 column prepared 5-18-80.

inositol for the various experiments performed. The poly-TMS derivatives of gulonic acid elute first as the hexa-TMS gulonic acid followed by presumably the pentasilylated species. EI spectra of both peaks are identical as TMS groups are cleaved quite readily. The poly-TMS derivatives of myo-inositol elute in three peaks. The first peak is identified as the penta-TMS derivative, followed by hexa-TMS-myo-inositol. The third peak was not characterized as the hexa-TMS peak was so intense that the GC/MS run was stopped to avoid damage to the filament. The presence of the desired compound was confirmed by identifying the molecular (parent) ion in the CI mass spectra. Much higher intensities at M-15 were usually observed, but expected, as the loss of a methyl group by the molecular ion is prominent in most TMS spectra. The second gulonate peak could not be confirmed by CI due to its low intensity. Figures 8, 9, and 10 present the CI mass spectra of the molecular ions of hexa-TMS-gulonic acid, penta-TMS-myo-inositol, and hexa-TMS-myo-inositol, respectively. The rather broad cluster about the molecular ion is primarily due to the three naturally occurring isotopes of silicon.

B. Oxygen-18 Labeling of L-gulonic Acid at the Carboxyl Group

As was discussed in the introduction, the saponification of L-gulono-γ-lactone results in the incorporation of an atom of oxygen-18 in the carboxyl group of L-gulonic acid. No ^{18}O exchange could occur with the alcohol at C-6 of L-gulonic acid. Therefore, any increase in m/z + 2 is due to an oxygen-18 atom in the carboxyl



M + H = 629

Figure 8: CI Spectrum of the Molecular Ion of 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-L-gulonic Acid.

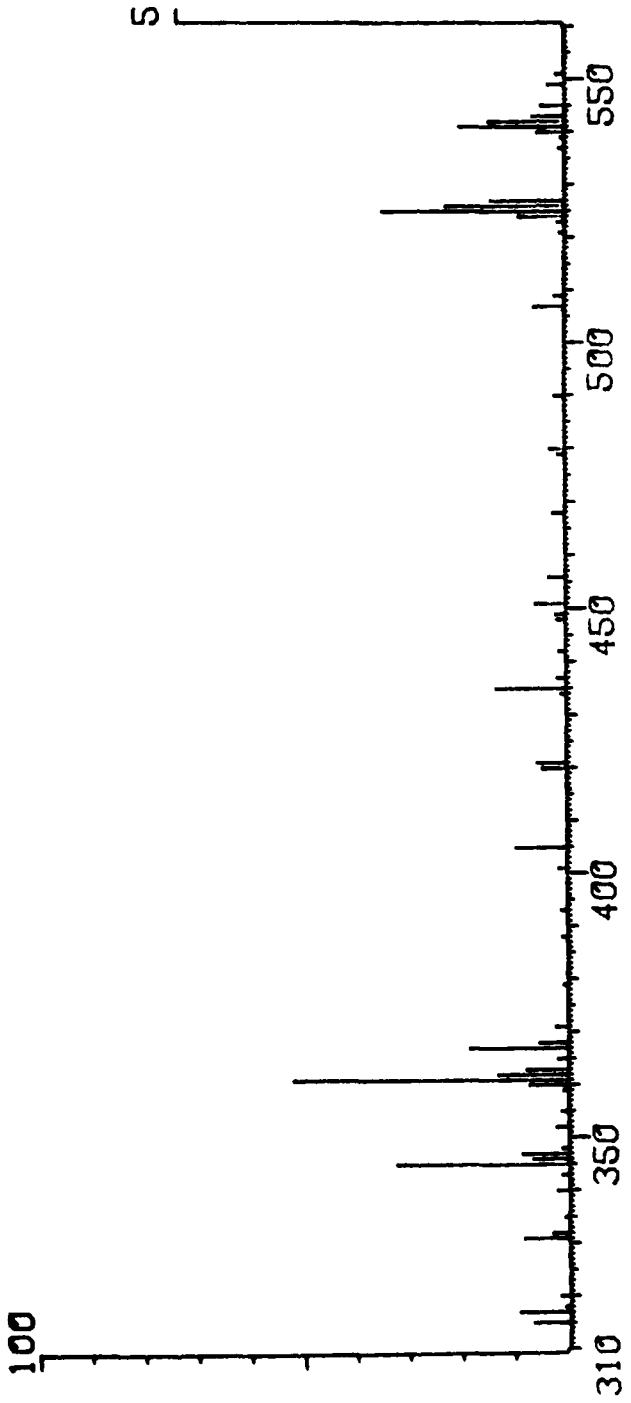
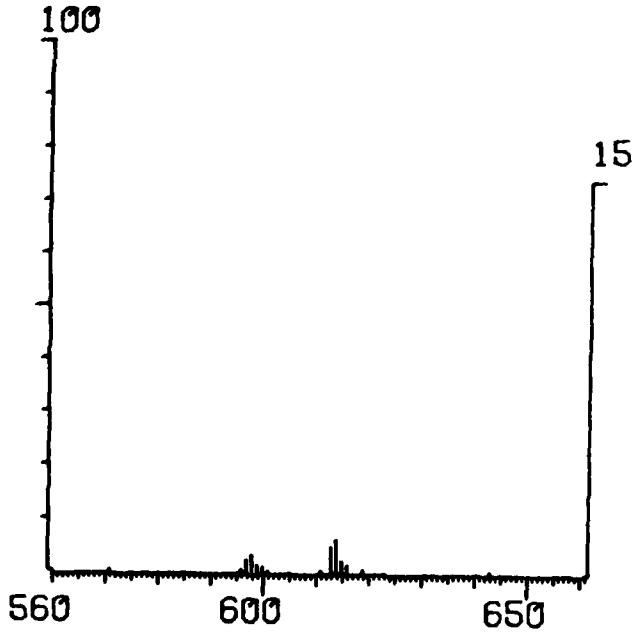


Figure 9: CI Spectrum of the Molecular Ion of Pentakis-O-(trimethylsilyl)-myo-inositol.



M + H = 613

Figure 10: CI Spectrum of the Molecular Ion of 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-myo-inositol.

fragment. A visual comparison of the standard hexa-TMS-gulonic acid with the ^{18}O labeled hexa-TMS-gulonic acid indicates that labeled carboxyl fragments occur at several m/z values, with especially well-defined ions (m/z and m/z + 2) at 292, 333, 359, 423, 433, and 435 in the EI spectrum. Confirmation of this inspection was achieved by comparing the ratios of the peak areas (m/z + 2 : m/z) defined by the trace of a single ion in its reconstructed ion chromatogram (RIC). Some results are listed in Table 5. Ions 319 and 333 were selected for comparison by single ion monitoring for two reasons. Both ions occur with approximately the same relative intensity. More importantly, the ions are fairly large fragments which reduces the possibility of other fragments of the same m/z ratio contaminating the results. As expected, a comparison of the ratios of the areas (Table 6) conclusively show that m/z 333 contains the ^{18}O labeled carboxyl group while the m/z 319 fragment is not labeled. Appendix I lists the intensities of all relevant ions of the carboxyl labeled TMS derivative. Using an analysis similar to that discussed later, it can be shown from this data that the fragments 292, 333, 423, 433, and 613 have $20 \pm 2\%$ excess ^{18}O in their corresponding m/z + 2 values. However, the fragment at m/z 319 shows no excess ^{18}O at m/z 321.

Table 5: Comparison of $\frac{m/z + 2}{m/z}$ Ratio of Areas of Oxygen-18 Labeled L-gulonic Acid vs. Unlabeled L-gulonic Acid.

$\frac{m/z + 2}{m/z}$	Unlabeled L-gulonic Acid (a) (%)	^{18}Carboxyl O-Labeled L-gulonic Acid (a) (%)	Natural Abundance (b) (%)
119/117 (d)	6.6	7.2	4.08
321/319 (d)	17.1	17.2	14.82
335/333 (d)	16.0	38.9	15.03
437/435 (d)	17.5 (c)	31.0	21.87

(a) poly TMS derivative
(b) provided by CAMFAC (library computer program)
(c) this ratio is lower due to an overlap of m/z 433 (see Figure 7)
(d) areas derived by reconstructing single ion traces from full mass spectral scans (m/z 100 to m/z 650)

Table 6: Comparison of m/z 319 and 333 by Single Ion Monitoring

m/z + 2 / m/z	Unlabeled L-gulonic Acid (a) (%)	^{18}O-Labeled Carboxyl L-gulonic Acid (a) (%)	Natural Abundance (b) (%)
321/319 (c)	12.7	14.2	14.82
335/333 (c)	13.7	35.6	15.03

(a) poly-TMS derivative (derived from gulonolactone)
(b) provided by CAMFAC (library computer program)
(c) ratios given as percentage of the areas of the relevant gas chromatographic traces for individual ions using single ion monitoring

C. Oxygen-18 Exchange at C-1 of D-glucuronic Acid

Since it is possible that oxygen-18 exchange with the solvent may occur at the C-1 position (aldehyde) of D-glucuronic acid and distort the enzymatic results, it is necessary to determine how much exchange occurs. D-glucuronic acid was incubated with ^{18}O enriched water at the same buffer and temperature conditions as the enzymatic reaction. At various incubation times, D-glucuronic acid was chemically reduced to L-gulonic acid, thereby locking in any ^{18}O that had exchanged. A comparison of the ratios of the areas produced by the RIC trace was undertaken for those EI ion fragments not containing the carboxyl group, i.e., m/z 117, 133, 205, and 319. The results (Table 7) indicate that ^{18}O exchange does not occur to any significant degree. Single ion monitoring (Table 8) of m/z 205, 207, 319 and 321 shows similar results. Additionally, no increase in any m/z + 2 for the remaining peaks in the EI spectra was noted, even with incubation up to 60 minutes.

Attempts to force ^{18}O exchange resulted in a breakdown of D-glucuronic acid. However, mass spectral analysis of the resulting unknown compounds did not indicate that any ^{18}O exchange had occurred. Therefore, it is believed that ^{18}O exchange probably does not occur at the reaction conditions and will not affect the enzymatic results.

The oxygen-18 enriched water used for the above experiments was analyzed by low resolution mass spectrometry for the amount of ^{18}O enrichment and estimated to be approximately 10-14%. However,

Table 7: Comparison of m/z + 2 : m/z Ratios of ^{18}O Exchange Experiments on D-glucuronic Acid (a,d)

Technique	$\frac{m/z + 2}{m/z}$	Control (%)	^{18}O Label x 1 min (%)	^{18}O Label x 5 min (%)	^{18}O Label x 20 min (%)	Natural Abundance (b) (%)
Integration of Total Peak Area (1st GC peak and 2nd GC peak)	119/117	10.1	11.3	12.4	10.9	4.1
	135/133	11.4	15.1	13.4	12.1	4.2
	207/205	11.9	13.8	13.7	12.6	8.7
Area Ratios of 1st GC Peak (c)	119/117	10.4	11.6	12.4	10.9	4.1
	135/133	12.3	16.6	13.6	12.1	4.2
	207/205	12.1	13.7	13.5	12.5	8.7
Area Ratios of 2nd GC Peak (c)	119/117	10.0	11.5	11.8	10.9	4.1
	135/133	11.4	18.3	15.1	13.0	4.2
	207/205	11.7	14.9	14.7	13.4	8.7

(a) all compounds were analyzed as the poly-TMS gulonic acid derivative
(b) provided by CAMFAC (library computer program)
(c) see retention times, Table 3
(d) ratios calculated from the areas of the RIC traces

Table 8: Comparison of m/z 205 and 319 by Single Ion Monitoring

$\frac{m/z + 2}{m/z}$	Control (a) (%)	^{18}O-Labeled x 20 min (a) (%)	Natural Abundance (b) (%)
207/205 (c)	10.7	11.6	8.7
321/319 (c)	13.4	14.8	14.8
207/205 (d)	12.2	13.1	8.7
321/319 (d)	11.5	13.6	14.8

(a) all compounds were analyzed as the poly-TMS gulonic acid derivative (derived from D-glucuronic acid)
(b) provided by CAMFAC (library computer program)
(c) area ratios of 1st GC peak, see Table 3
(d) area ratios of 2nd GC peak, see Table 3

the assay provided by the supplier listed the enrichment at 20.1%. Since approximately 20% enrichment was observed in the carboxyl labeling experiments, it is thought that the lower analysis may be due to water condensation in the mass spectrometer.

D. Enzymatic Oxidation-Reduction of Myo-inositol in the Presence of $^{18}O_2$

Although the crude extract of myo-inositol oxygenase was somewhat less active than Channa Reddy's preparation at the same stage (8), sufficient activity (51.5 μ Katals/Kg) was measured. Protein concentration of the ASD fraction was 25.78 mg/ml. After storage for one month at -10°C, the enzyme had lost all activity. However, the addition of cysteine and iron brought back some activity. Activity could also be restored after more than two months storage at -10°C. Table 9 summarizes the results of these assays. D-glucuronic acid reductase was also assayed at the crude stage and found to have a much higher specific activity than the oxygenase (1.10 m Katals/Kg). Activity decreased slightly after prolonged storage.

In a preliminary experiment, the enzymatic cleavage was carried out with $^{18}O_2$ generated from 20.0% ^{18}O enriched water. When compared to its standard, results (Table 10) indicate that the carboxyl group of L-gulonic acid contained the label. Furthermore, no label was seen at C-6 (original aldehyde position) as indicated by the 321/319 ratio. However, only a 10% increase in the area ratio of m/z 335 to m/z 333 was noted. This could occur if the generated oxygen-18 gas was contaminated with normal isotopic oxygen and/or if the enrichment

Table 9: Summary of Myo-inositol Oxygenase Assays

Protein ID	Specific Activity (μKatals/Kg)	Total Activity (μKatals)
Crude extract - UCS (a)	I. 13.9 II. 12.5	I. .519 II. .467
Ammonium Sulfate Fractionation - ASD (b)	I. 51.5 II. 45.6	I. .179 II. .159
After 36 days storage at -10°C - ASD (b)	I. 1.8 II. 18.3	---- ----
After 76 days storage at -10°C - ASD (b)	I. 0 II. 16.2	---- ----

I. assayed with no cofactors present
II. assayed with 2.0 mM cysteine and 1.0 mM Fe^{++}
(a) assayed in sodium acetate buffer, pH 6.0
(b) assayed in sodium phosphate buffer, pH 7.2.

Table 10: Comparison of Enzyme Systems Carried out with Generated $^{18}O_2$ and Normal Isotopic O_2

$\frac{m/z + 2}{m/z}$	Enzyme-Standard (a) (%)	Enzyme-^{18}O Label (a,d) (%)	Natural Abundance (b) (%)
321/319 (c)	14.17	14.95	14.82
335/333 (c)	14.86	24.88	15.03

(a) all compounds were analyzed as the poly-TMS gulonic acid derivative
(b) provided by CAMFAC (library computer program)
(c) ratios given as a percentage of the areas of the RIC traces
(d) $^{18}O_2$ generated from 20.0% oxygen-18 enriched water

of water was actually less than 20.0%. Of more importance, the possibility existed that the enzyme was not completely precipitated and separated from the reaction mixture. Therefore, live enzyme could have continued to cleave to myo-inositol during the extraction process in the presence of normal isotopic oxygen. This would account for the lower results. Therefore, in subsequent runs, the enzyme was killed by boiling in a hot water bath for approximately five minutes prior to the extraction process.

The enzymatic cleavage in 90% $^{18}O_2$ produced distinct results. The oxygen enriched gas was analyzed by low resolution mass spectrometry at the completion of the second enzymatic reaction. The ratio of $^{16}O_2$: $^{16}O^{18}O$: $^{18}O_2$ was approximately 1.7% : 14.5% : 83.7% compared to a calculated ratio from 90% $^{18}O_2$ of 1% : 18% : 81% (see Table 11). Furthermore, the amount of enrichment was calculated to be 91.8 $\pm$ .1%. Thus, little or no contamination of the enriched gas occurred during the enzymatic experiments.

The presence of ^{18}O can be clearly seen in the fragments containing the carboxyl group and no label appeared at any other position. Additionally, only one atom of ^{18}O was incorporated during the cleavage. Figure 11 presents a comparison of the EI spectra of the labeled gulonic acid (hexa-TMS derivative) to a standard. The reconstructed gas chromatograms for the standard and the two enzymatic ^{18}O labeled reactions are reproduced in Figure 12. A calculation of the areas of the RIC's for m/z 319, 321, 333, 335, 613, and 615 presents evidence that the carboxyl

Table 11: Assay of Isotopic Content of ^{18}O Enriched O_2 by Low Resolution Mass Spectrometry

		Background (a)	Assay of Sample (a)	Corrected (b)	Ratio (%)
Completion of 1st Enzyme Run	$^{14}N_2$	1.00	1.00	----	----
	$^{16}O_2$	.22	.15	----	----
	$^{16}O^{18}O$	----	.22	.22	15.3
	$^{18}O_2$	----	1.22	1.22	84.7
Start of 2nd Enzyme Run	$^{14}N_2$	1.00	1.00	----	----
	$^{16}O_2$	.21	.23	.02	2.1
	$^{16}O^{18}O$	----	.14	.14	15.1
	$^{18}O_2$	----	.77	.77	82.8
Completion of 2nd Enzyme Run	$^{14}N_2$	1.00	1.00	----	----
	$^{16}O_2$	.23	.28	.05	1.7
	$^{16}O^{18}O$	----	.42	.42	14.5
	$^{18}O_2$	----	2.42	2.42	83.7
Control-Air Sample	$^{14}N_2$	1.00	----	----	----
	$^{16}O_2$	.24	----	----	----

Table 11: continued

(a) data shown is relative to the height of $^{14}N_2$ peak
(b) corrected by subtraction of relative $^{16}O_2$ background

Notes: 1. Average ratio of $^{16}O^{18}O/^{18}O_2$ is 17.9%

2. Amount of ^{18}O enrichment is 91.8 $\pm$ .1%. This is calculated from the average ratio of $^{16}O^{18}O/^{18}O_2$ by the following formula:

$$2[X(1-X)]/X^2 = .179$$

where X is the amount of ^{18}O enrichment and (1-X) is the amount of ^{16}O present

Figure 11: Comparison of the EI Spectra of 90% ^{18}O Carboxyl Labeled 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-L-gulonic Acid and an Unlabeled Standard.

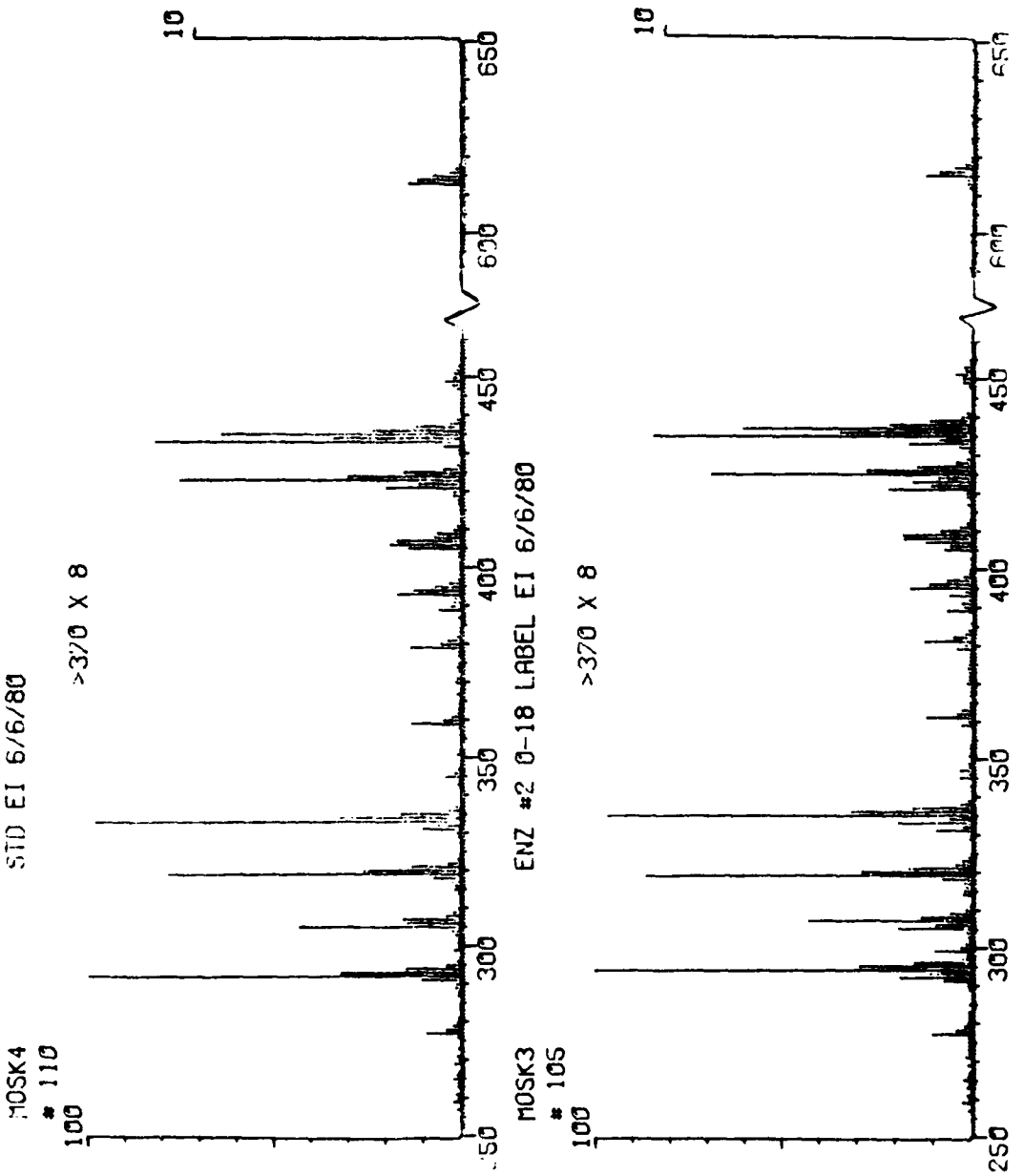
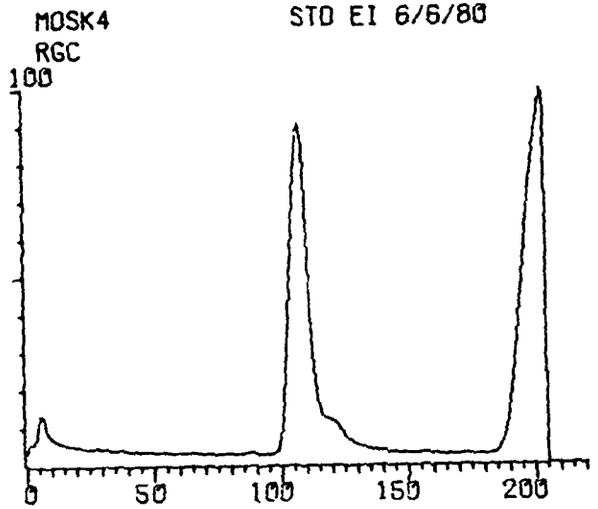
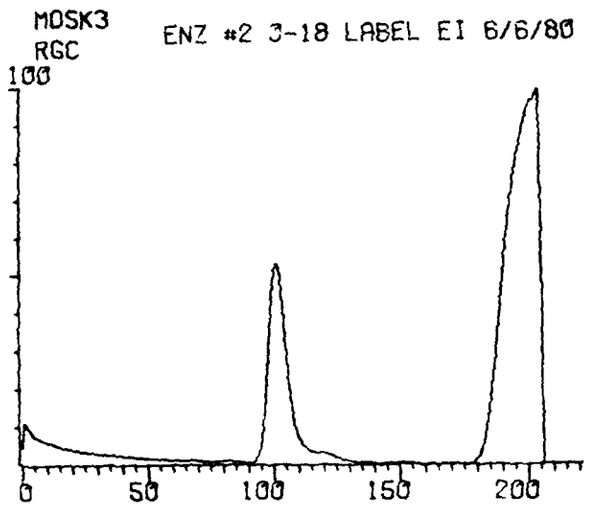
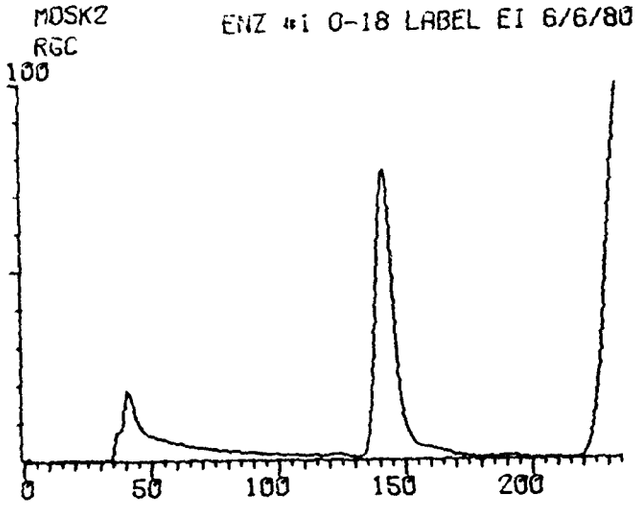

MOSK4
* 110
STD EI 6/6/80
>370 X 8
MOSK3
* 105
ENZ #2 0-18 LABEL EI 6/6/80
>370 X 8

Figure 12: Reconstructed Gas Chromatographs of the Unlabeled Standard Enzymatic Reaction and the ^{18}O Labeled Enzymatic Reactions.


MOSK4
RGC
STD EI 6/6/80
100
0
50
100
150
200


MOSK3
RGC
ENZ #2 3-18 LABEL EI 6/6/80
100
0
50
100
150
200


MOSK2
RGC
ENZ #1 0-18 LABEL EI 6/6/80
100
0
50
100
150
200

group contains the label and only one atom of ^{18}O is incorporated (see Table 12). The percentage of oxygen-18 is calculated from the following formula (25):

$$\% \ ^{18}O \text{ excess} = \frac{(m+2)_e - [(m+2)_s/m_s] \times m_e}{m_e + (m+2)_e - [(m+2)_s/m_s] \times m_e} \times 100$$

where m is intensity from non-isotopic peak

m+2 is intensity from peak 2 amu above m

s refers to data from the unlabeled standard

e refers to data from the labeled enzyme runs

This calculation subtracts the intensity of ^{16}O and normal isotopic ^{18}O which contributes to the intensity of the m/z+2 peak. The subtraction also takes into account any other fragment which may be overlapping the m/z+2 peak if the fragment is not present in the m/z peak of interest. When there is a peak (or peaks) just prior to the ion of interest, the raw data for the intensity of m must be corrected. This is accomplished by the relationship for m/z 292, 433:

$$(m_n)_{corr} = m_n - (y_n)(m+1)_s/m_s$$

or for m/z 333, 423:

$$(m_n)_{corr} = m_n - (y'_n)(m+2)_s/m_s$$

Table 12: Areas (a) of RIC's for Ions of Interest and Percent of Oxygen-18 Excess (b)

Ion of Interest	% Area of m_s	% Area of $(m+2)_s$	Enzyme Run	% Area of M_e	% Area of $(m+2)_e$	% ^{18}O Excess
613,615	67.47	32.53	#1	12.18	87.82	87.1
			#2	14.12	85.88	84.8
333,335	86.16	13.84	#1	16.74	83.26	82.8
			#2	17.67	82.33	81.8
319,321	85.26	14.74	#1	82.31	17.69	4.0
			#2	83.81	16.19	2.0

(a) areas are listed as a percentage of the total area (m/z plus $m/z+2$)
(b) see formula, page 55.

Table 13: Calculated Percentage of ^{18}O Excess in Labeled Enzyme Runs #1 and #2

Ion of Interest	Relative Intensity of [a] m_s	Relative Intensity of [a] $(m+2)_s$	Enzyme Run #1	Relative Intensity of [a] m_e	Relative Intensity of [a] $(m+2)_e$	$\%^{18}O$ Excess
613,615	100.00	50.23	#1	11.94	100.00	88.7
			#2	14.02	100.00	86.9
433,435 (corrected) [b]	97.56	78.38	#1	15.51	100.00	84.9
			#2	17.89	100.00	82.7
423,425 (corrected) [b]	87.38	19.52	#1	12.04	74.17	85.6
			#2	13.49	82.59	85.5
333,335 (corrected) [b]	96.04	15.71	#1	18.52	100.00	84.0
			#2	18.15	96.86	83.8
292,294 (corrected) [b]	96.66	15.25	#1	16.32	98.44	85.5
			#2	16.74	100.00	85.3
319,321	78.75	13.61	#1	84.75	17.61	3.4
			#2	87.06	17.09	2.3

(a) see Appendix II
(b) see formula, page 55.

where y (y') is the intensity of the peak 1 amu (2amu) prior to the ion of interest and n can be s or e. This assumes that the prior peak does not have ^{18}O in excess. Table 13 presents the calculated amount of oxygen-18 excess of various ions of interest. From the data given in Tables 11 and 13 the amount of ^{18}O incorporation per molecule of D-glucuronic acid is 0.97 atoms from O_2. Appendix II lists the intensities of all relevant ions. These results clearly indicate that only one atom of ^{18}O is incorporated at the carboxyl group during the enzymatic cleavage of myo-inositol to D-glucuronic acid and that no exchange occurs.

IV. DISCUSSION

The various oxygen-18 labeling studies which are described here help to further clarify the mechanism of myo-inositol oxygenase. Since the oxygen-18 label did not appear at the aldehyde site of D-glucuronic acid, mechanisms III, IV, and V proposed by Dr. Hamilton (Figure 4) can be eliminated. Mechanisms I and II still remain as feasible pathways since incorporation of an oxygen atom at the carboxyl group can occur by either mechanism. Work is still continuing in this laboratory to further define these mechanisms.

A unique result of this research was that the ion M-15 (m/z 613) was observed in the EI spectra of 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-L-gulonic acid. Polysilylated ethers fragment quite readily and the observation of such a large fragment is a rarity. A literature search of similar polysilylated compounds produced no compound with fragment ions above m/z 450. The observation of M-15 eliminated the need for CI spectra of the 90% ^{18}O labeled compounds and the corresponding unlabeled standard.

In the course of this work, it was found that the sodium salt of L-gulonic acid was extremely hydroscopic and formed a thick gummy oil. For this reason, care was taken to keep all products dry. Therefore, the salts were dried and stored in a vacuum dessicator and silylated immediately after removal from

the dessicator. Once the oil had formed, the salt could not be silylated. Attempts to dissolve the oil in pyridine with heating also failed.

In the enzymatic reactions, it was originally thought that L-gulonic acid could be separated and concentrated by using an ion-exchange resin according to the method of Ishidate, Matsui, and Okada (26). Although standard solutions of myo-inositol and sodium gulonate could be separated, the enzymatic mixture remained at the top of the column. Therefore, the alternate method of separation by gas chromatography was used with excellent results.

Future work in the oxygen-18 labeling study of the proposed mechanisms should include enzymatic runs in the presence of ^{18}O enriched water. Although Charalampous reported no incorporation of ^{18}O from enriched water (1), the newer GC/MS techniques used in this research would confirm his results. Additionally, if an ^{18}O label could be placed in the aldehyde group of D-glucuronic acid and reduced to L-gulonic acid, one would expect to see a definite relocation of the ^{18}O labeled fragments.

The goal of this research was to further define the mechanism of myo-inositol oxygenase by determining the location and number of oxygen atoms incorporated during the cleavage of myo-inositol to D-glucuronic acid. It was hoped that from such a study several proposed mechanisms could be eliminated. This was accomplished by the various oxygen-18 labeling experiments described in this work.

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APPENDIX I

INTENSITIES OF RELEVANT IONS OF CARBOXYL LABELED TMS DERIVATIVE

CARBOXYL LABEL 18 O
SPEC.# 83
AMP. : 137264

m/z	Intensities (%) [a]						
272	.10	.27	.15	.23	.27	13.62	4.52
279	2.61	.48	.32	.00	.00	.04	.39
286	.01	.29	.09	.46	.53	8.95	100.00
293	29.96	36.40	9.49	3.81	.78	.09	.09
300	.00	.16	.04	.50	1.44	38.80	12.90
307	25.37	6.87	3.17	.48	.11	.00	.00
314	.13	1.78	.66	1.70	3.04	94.89	29.19
321	16.16	3.67	1.20	.36	.13	.01	.09
328	.09	.13	.15	10.30	4.69	92.00	31.26
335	35.47	9.97	4.16	.94	.19	.00	.00
342	.00	.53	.25	4.35	1.32	1.45	.40
349	.18	.00	.60	.10	.00	.15	.04
356	.00	.04	.18	11.73	3.82	4.51	1.20
363	.48	.15	.13	.06	.34	.13	.02
370	.00	.00	.00				

CARBOXYL LABEL 18 O
M/E 613
AMP. : 9920

m/z							
372	.16	.16	.48	.48	.00	.96	.96
379	17.41	5.32	7.09	1.12	.32	.96	.48
386	.00	.96	.48	8.54	1.77	2.41	.48
393	21.45	15.96	13.87	5.96	3.38	1.45	.16
400	.16	.00	.16	.16	.96	19.03	23.38
407	24.83	13.70	12.25	3.06	1.77	.32	.16
414	.48	.48	.00	.96	.96	2.09	.48
421	32.58	14.83	95.96	38.87	40.48	12.58	6.61
428	1.45	.16	.16	.00	.48	95.00	40.64
435	100.00	37.58	32.58	10.64	3.87	.32	.48
442	.32	.16	.48	.48	.00	1.93	2.41
449	5.00	1.12	2.41	.48	.16	.48	.48
456	.00	.48	.48	.16	.16	.00	.32
463	.16	.96	.48	.00	3.06	1.45	.96
470	.16	.00	.32				

CARBOXYL LABEL 18 O
SPEC.# 83
AMP. : 1472

m/z							
608	.00	.00	.00	.00	.00	100.00	93.47
615	82.60	40.21	21.73	8.69	.00	.00	

(a) Intensities can be normalized in each set of data by multiplying the amplitude by the % intensity reported.

(b) Data represents the summation of the intensities of five MS scans about the center of the reconstructed gas chromatogram.

APPENDIX II

INTENSITIES OF RELEVANT IONS OF ENZYMATICALLY PRODUCED L-GULONIC ACID

A. Unlabeled Standard (TMS derivative)

MOSK4
STD EI 6/6/80
SPEC.# 110
AMP. : 432396

m/z	Intensities (%)[a]						
284	.12	.21	.09	.39	.44	1.12	1.65
291	10.51	100.00	31.74	15.25	3.37	.91	.16
298	.19	1.64	.49	.48	.55	1.19	2.52
305	43.02	14.77	15.59	4.28	1.92	.45	.13
312	.09	.19	.49	1.81	1.26	2.36	7.02
319	78.75	25.93	13.61	3.05	.91	.28	.11
326	.05	.18	.24	.44	1.08	10.24	6.54
333	97.69	32.90	15.71	3.57	1.03	.30	.11
340	.03	.14	.18	.91	.43	4.35	1.46
347	.78	.27	.21	.17	.82	.36	.26
354	.16	.13	.18	.25	.54	13.35	4.48
361	2.44	.64	.36	.12			

>

STD EI 6/6/80
SPEC.# 110
AMP. : 44206

m/z							
383	.39	.23	.27	.20	.47	.30	7.33
390	3.22	2.51	1.98	21.33	15.18	8.87	3.28
397	.96	.33	.32	.09	.27	.28	.51
404	.79	17.26	23.80	21.05	9.18	7.68	2.71
411	1.14	.23	.30	.21	.25	.13	.58
418	.51	2.59	2.18	25.10	13.78	92.67	36.52
425	19.52	5.36	1.56	.42	.30	.44	.76
432	5.78	100.00	42.23	78.38	29.35	14.36	3.89
439	1.19	.39	.26	.25	.11	.33	.21
446	.20	1.57	2.83	5.39	2.34	2.18	.88
453	.54	.27	.23	.26	.07	.18	.06
460	.16	.11	.09	.28	.14	.19	.45
467	3.41	1.75	.82	.44	.25	.16	.16

>

STD EI 6/6/80
SPEC.# 110
AMP. : 3024

m/z							
493	1.38	3.50	2.05	38.69	27.05	27.31	12.43
497	8.66	3.76	3.76	1.32			

>

STD EI 6/6/80
SPEC.# 110
AMP. : 7544

610	1.24	1.80	2.33	100.00	83.80	50.23	21.71
617	8.43	3.44	.79	.66			

(a) Intensities can be normalized in each set of data by multiplying the amplitude by the % intensity reported.

(b) Data represents the summation of the intensities of five MS scans about the center of the reconstructed gas chromatogram.

B. ^{18}O Labeled-Enzyme Run #1

MOSK2
ENZ #1 O-18 LABEL EI 6/6/80
SPEC.# 141
AMP. : 31616

m/z	Intensities (%)[a]						
281	.60	.40	.34	.00	.26	.13	.20
288	.12	.67	.94	7.42	18.68	11.13	98.44
295	31.17	15.71	3.23	.60	2.08	.88	.58
302	.06	.74	1.55	15.92	7.89	44.81	14.37
309	6.94	1.41	.48	.13	.06	.60	2.08
316	1.21	2.56	5.80	84.75	27.93	17.61	4.25
323	2.56	.74	.32	.20	.00	.20	.40
330	.40	9.04	4.25	19.97	9.59	100.00	33.60
337	16.47	3.78	.87	.27	.06	.00	.40
344	.20	2.83	1.13	3.64	.93	.54	.32
351	.67	.07	.54	.40	.07	.00	.26
358	.26	2.70	1.27	11.74	4.12	2.49	.60
365	.48	.06	.34	.26	.20	.00	.06
372	.12	.00	.00	.06	.00	.10	.00
379	.50	.20					

ENZ #1 O-18 LABEL EI 6/6/80
SPEC.# 141
AMP. : 3392

m/z							
414	.00	.00	.00	.00	.29	.58	1.59
421	24.82	12.26	17.27	9.43	74.17	31.42	16.68
428	3.77	1.59	.00	.00	1.23	16.03	8.49
435	100.00	40.86	71.34	27.65	14.15	3.41	.94
442	.29	.00	.29	.00	.94	.00	.29
449	1.88	2.18	5.01	1.53	1.23	.29	.00
456	.29	.00	.94	.00	.00	.00	.00
463	.00	.00					

>

ENZ #1 O-18 LABEL EI 6/6/80
RGC
AMP. : 2026

m/z							
610	1.48	.98	.98	11.94	15.10	100.00	93.58
617	64.06	26.65	13.36	3.55	.98	2.56	.00
624	.98	.98	3.55	2.56	.98	1.43	.98

(a) Intensities can be normalized in each set of data by multiplying the amplitude by the % intensity reported.

(b) Data represents the summation of the intensities of five MS scans about the center of the reconstructed gas chromatogram.

C. ^{18}O Labeled-Enzyme Run #2

MOSK3
ENZ #2 0-18 LABEL EI 6/6/80
SPEC.# 105
AMP. : 260168

m/z	Intensities (%)[a]						
285	.35	.22	.28	.25	.82	.92	7.89
292	19.24	11.61	100.00	30.28	14.69	2.97	1.29
299	10.09	2.70	1.66	.61	1.33	1.66	20.31
306	9.51	43.73	13.21	6.24	1.41	.49	.19
313	.27	2.21	2.58	1.61	2.61	7.69	87.06
320	28.72	17.09	4.20	2.77	.80	.38	.14
327	.19	.28	.37	.54	9.57	4.40	19.69
334	9.65	96.86	32.40	15.48	3.49	.97	.28
341	.13	.16	.54	.33	3.07	1.17	3.30
348	1.09	.72	.36	.76	.36	.61	.45
355	.20	.15	.25	.22	2.93	1.53	12.29
362	4.12	2.42	.68	.48	.17	.74	.39
369	.40	.18	.14	.12	.15	.13	.13

>

ENZ #2 0-18 LABEL EI 6/6/80
SPEC.# 105
AMP. : 27460

m/z							
415	.33	.22	.79	.33	1.69	1.56	26.78
422	12.25	19.13	10.50	82.59	32.48	17.37	4.45
429	1.69	.29	.51	4.22	19.67	10.84	100.00
436	41.77	72.00	26.16	12.98	3.24	1.21	.29
443	.37	.33	.10	.37	.37	1.11	2.40
450	3.05	5.07	2.12	2.08	.56	.64	

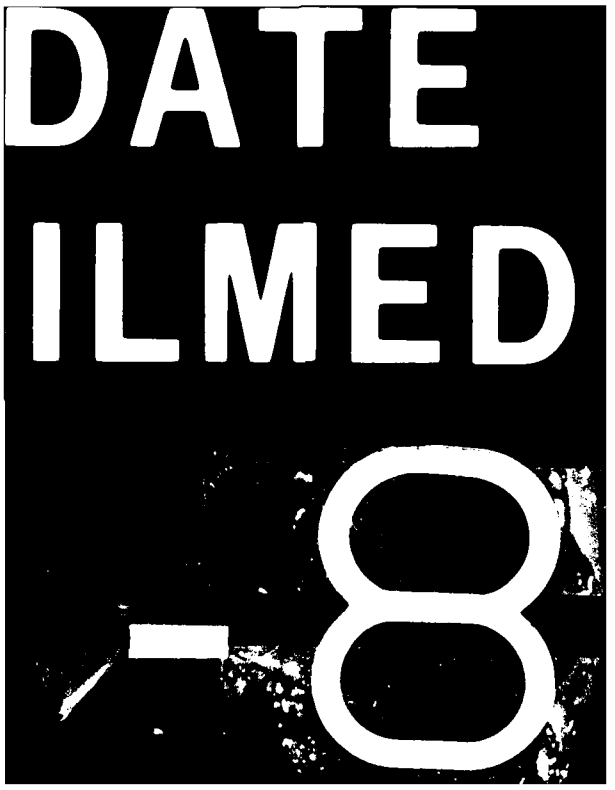
>

ENZ #2 0-18 LABEL EI 6/6/80
SPEC.# 105
AMP. : 4134

m/z							
611	1.79	5.80	14.02	12.19	100.00	69.27	44.17
618	17.65	6.82	2.75	.48			

(a) Intensities can be normalized in each set of data by multiplying the amplitude by the % intensity reported.

(b) Data represents the summation of the intensities of five MS scans about the center of the reconstructed gas chromatogram.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